REMARKS

Claims 1-11, 13-22 and 24-47 are currently presented for examination. Claims 136-143 have been added and claims 1 and 45 have been amended. Neither the new claims nor the claim amendments constitute addition of new matter to the instant application.

Support for each of the new claims and claim amendments can be found in the claims as originally filed and throughout the specification. In particular, support for the amendment to claim 1 can be found on page 40, line 21 to page 45, line 2; page 98, line 6 to page 103, line 22 (Example 7); the claims as originally filed and elsewhere throughout the specification. Support for new claims 136-143 can be found on page 44, lines 1-13; page 45, lines 22-28; page 46, line 28 to page 47, line 21; page 51, line 23 to page 52, line 13; page 56, line 14 to page 57, line 10; page 76, line 1 to page 78, line 17; page 103, line 24 to page 109, line 15 (Example 8) and elsewhere throughout the specification. Accordingly, no new matter has been added to the application.

Applicants have reviewed the rejections of claims 1-11, 13-22 and 24-47 as set out in the instant Office Action. After careful consideration, Applicants respectfully traverse these rejections.

Information Disclosure Statement

Applicants would like to draw the Examiner's attention to the Information Disclosure Statement submitted herewith, which includes an Office Action from copending U.S. Patent Application Number 09/948,993.

Objection to the Specification

The specification of the instant application is objected to because it contains an embedded hyperlink at page 54. The Examiner has requested that Applicants delete all browser executable code contained within the application.

Applicants have searched the specification and found browser executable code occurring on pages 52-54 and 119. In some instances, Applicants have deleted the text containing the browser executable code. In other instances, Applicants have replaced the text containing the browser executable code with text that teaches the public how to access the intended website by

using a web browser. Accordingly, the amended specification no longer contains browser executable code.

In view of the above amendments, Applicants respectfully request the Examiner to withdraw the objection to the specification.

Other Amendment to the Specification

Applicants have amended the specification to correct a typographical error at page 50, line 10. In particular, the Granger *et al.*, Published PCT Application No. WO 98/01579, was inadvertently cited as WO 98/01879. Applicants have amended the paragraph beginning at page 49, line 19 and ending at page 50, line 13 to correct this error.

Rejection of Claims 1-11, 25, 28-40 and 42-47 Under 35 U.S.C. § 112, Second Paragraph

The Examiner rejects claims 1-11, 25, 28-40 and 42-47 under 35 U.S.C. § 112, second paragraph as failing to particularly point out and distinctly claim subject matter that is regarded as an invention. In particular, the Examiner asserts that the phrase "said fusion promoter comprising at least one promoter that is modified to have altered activity in at least one gram-positive organism" is allegedly vague and indefinite because the claim allegedly neither sets forth how the promoter is "modified" nor the type of "altered activity" that results. In addition, with respect to Claim 45, the Examiner alleges that a word is missing prior to the word "microbe."

Applicants maintain that Claims 1-11, 25, 28-40 and 42-47 are not indefinite; however, solely to expedite the allowance of these claims, Applicants have replaced the phrase "that is modified to have altered activity" in original claim 1 (and by dependence, claims 2-11, 25, 28-40 and 42-47), with the phrase "comprising at least one nucleotide sequence modification which alters the transcriptional activity of said promoter." Applicants respectfully submit that this amendment addresses the Examiner's concerns regarding the use of the term "modified" and the phrase "altered activity."

In addition to the foregoing amendment, Applicants have corrected the typographical error in claim 45 by adding the word "a" just prior to the word "microbe." This amendment is in accordance with the Examiner's reading of the claim as stated on page 5 of the instant Office Action.

In view of the amendments to claims 1 and 45, Applicants request that the Examiner withdraw the rejection of claims 1-11, 25, 28-40 and 42-47 under 35 U.S.C. § 112, second paragraph.

Rejection of Claim 28 Under 35 U.S.C. § 112, First Paragraph

The Examiner rejects claim 28 under 35 U.S.C. § 112, first paragraph as containing subject matter that was not described in the specification in such a way as to enable a skilled artisan to make and/or use the claimed subject matter. In particular, the Examiner asserts that "it is not clear that all of the plasmids [named in claim 28] are readily available to the public."

Applicants respectfully submit that each of the plasmids named in claim 28 are known and readily available to the public. Applicants note claim 28 recites a vector "comprising at least one replicon selected from the group consisting of p15a, pC194 and pCT1138." Thus, the vectors set forth in claim 28 comprise an origin of replication that is equivalent to the origin of replication form one or more of the plasmids p15a, pC194 or pCT1138. Each of the named plasmids, p15a, pC194 and pCT1138 were known and available to the public at the time of filing the instant application. Moreover, each of these plasmids are still currently known and still publicly available.

Examples of acceptable relevant evidence that can be used to demonstrate that a biological material is known and available to the public are listed in the M.P.E.P.. In particular, M.P.E.P. § 2404.01 states that evidence relevant to demonstrate that a biological material is known and readily available to the public includes a showing of commercial availability, reference to the biological materials in printed publications, declarations of accessibility by those working in the field, evidence of predictable isolation techniques, or an existing deposit made in accordance with the rules set out in 37 C.F.R. § 1.801-1.809. (see M.P.E.P. § 2404.01).

To demonstrate that each of the vectors named in claim 28 are known and available to the public, Applicants provide herewith Exhibits A-F. Exhibit A shows that plasmid p15a was first isolated from *E. coli* in 1968. A reliable method for isolating this plasmid is described by Cozzarelli, N. R. et al. in *PNAS* **60**:992-999. Since its first isolation in 1968, p15a has been widely distributed among scientists and its replicon has been used in the construction of a variety of plasmid vectors. Many such vectors are commercially available. For example, the p15a replicon is contained in the plasmids pLysS and pLysE, both of which are available for purchase

from Novagen (see Exhibit B). In view of the foregoing evidence, which demonstrates the widespread commercial availability of p15a, its extensive use and a predicable method for its isolation, Applicants respectfully submit that p15a is known and available to the public.

Similar to p15a, the plasmid pC194 is known and available to the public. In particular, pC194 is a plasmid that was first isolated from *Staphylococcus aureus* and which has been in use since at least 1978 (see Exhibit C, see also Iordanescu, et al. *Plasmid* **1**:468-479). Exhibit D shows that pC194 is publicly available from the depository Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (hereinafter DSMZ), Braunschweig, Germany. DSMZ is an international depository authority recognized under the Budapest Treaty (see M.P.E.P. § 2405). In view of the foregoing evidence, Applicants respectfully submit that pC194 is known and available to the public.

The final plasmid named in claim 28, pCT1138, is also known and available to the public. The plasmid pCT1138, which is also known as the citrate plasmid, was originally isolated from *Lactococcus lactis* subsp. *lactis* (see Exhibit E; see also Pederson et al. *Mol. Gen. Genet.* **244**:374-382). Exhibit F shows that pCT1138 is publicly from the international depository authority, DSMZ. In view of the foregoing evidence, Applicants respectfully submit that pCT1138 is known and available to the public.

In view of the above arguments, which demonstrate that each of the plasmids named in claim 28 are known and available to the public, Applicants request that the Examiner withdraw the rejection of this claim under 35 U.S.C. § 112, first paragraph.

Rejection of Claims 1, 7, 9 and 10 Under 35 U.S.C. § 102(b)

The Examiner rejects claims 1, 7, 9 and 10 under 35 U.S.C. § 102(b) as being anticipated by published International Patent Application No. WO99/28508 (Marra et al.). In particular, the Examiner asserts that Marra et al. disclose an isolated nucleic acid comprising a fusion promoter having a promoter that is modified to have an altered activity in a gram-positive organism, wherein the promoter is linked to *tetO*. Furthermore, the Examiner asserts that Marra et al. disclose that the binding of a repressor to *tetO* represses transcription.

Applicants maintain that Marra et al. does not anticipate claim 1, which is independent, nor does it anticipate claims 7, 9 or 10, which are dependent on claim 1. In particular, Applicants point out that independent claim 1 recites, in relevant part, "an isolated nucleic acid comprising a

fusion promoter said fusion promoter comprising at least one promoter comprising at least one nucleotide sequence modification which alters the transcriptional activity of said promoter in at least one gram-positive organism said promoter being linked to at least one operator selected from the group consisting of *xylO, tetO, trpO, malO* and *λc1O* . . . ." The Examiner asserts that Marra et al. disclose fusion promoters comprising modified promoters linked to an operator sequence. After having carefully reviewed Marra et al., Applicants maintain that Marra et al. do not disclose a promoter which comprises a nucleotide sequence modification that alters the transcriptional activity of the promoter in a gram-positive organism. As such, Marra et al. does not disclose the claimed fusion promoters.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of claims 1, 7, 9 and 10 under 35 U.S.C. § 102(b).

Rejection of Claims 1-4, 7-11, 13-15, 18-22, 24-27, 29, 32, 33, 36-40 and 42-47 Under 35 U.S.C. § 103(a)

The Examiner rejects claims 1-4, 7-11, 13-15, 18-22, 24-27, 29, 32, 33, 36-40 and 42-47 under 35 U.S.C. § 103(a) as being obvious over published European Patent Application No. EP0186069 (Bujard et al.) in view of Sizemore, et al. (*J. Bacteriol.* **174**:3042-3048). In particular, the Examiner asserts that Bujard et al. disclose an isolated nucleic acid comprising a fusion promoter comprising the T5 promoter operatively linked to a *lac* operator and that Sizemore et al. allegedly disclose a xylose operon control region from *Staphylococcus xylosus*, which includes the *xyl* operator. The Examiner also asserts that it would have been obvious for a skilled artisan to combine the disclosures Bujard et al. and Sizemore et al. to achieve the subject matter of the above-mentioned claims because Bujard et al. suggest that the T5 promoter can be combined with any operator. The Examiner then asserts that a skilled artisan would have been motivated to combine these disclosures in view of the known equivalence of the *xyl* and *lac* operators and the known usefulness of the *xylO* operator.

Applicants maintain that claims 1-4, 7-11, 13-15, 18-22, 24-27, 29, 32, 33, 36-40 and 42-47 are not obvious because the combination of Bujard et al. and Sizemore et al. does not teach every element of these claims. Furthermore Applicants maintain that these claims are not obvious because, a skilled artisan would neither be motivated to combine the disclosure of Bujard et al. with Sizemore et al. nor would a skilled artisan reasonably expect a promoter from a

gram-negative organism fused to an operator from a gram-positive organism to successfully promote regulatable transcription in an organism. Each of these arguments are set out in detail below.

The above-rejected claims are not obvious because the combination of Bujard et al. and Sizemore et al. does not teach every element of these claims. For example, Claim 1 recites, in relevant part, a fusion promoter comprising "at least one nucleotide sequence modification which alters the transcriptional activity of said promoter in at least one gram-positive organism." Neither Bujard et al. nor Sizemore et al. disclose a promoter that comprises at least one nucleotide sequence modification which alters the transcriptional activity of the promoter in a gram-positive organism. As such, the combination of Bujard and Sizemore does not disclose every element of the rejected claims.

In addition to the foregoing, Applicants submit that claims 1-4, 7-11, 13-15, 18-22, 24-27, 29, 32, 33, 36-40 and 42-47 are not obvious because a skilled artisan would neither be motivated to combine the disclosure of Bujard et al. with Sizemore et al. nor would a skilled artisan reasonably expect a promoter from a gram-negative organism fused to an operator from a gram-positive organism to promote regulatable transcription in an organism. In particular, a fusion promoter construct that is based on the combination of Bujard et al. and Sizemore et al. would allegedly be a fusion promoter having a T5 promoter operably linked to a *xyl* operator. Applicants note that the T5 promoter is a coliphage promoter. Coliphage promoters are functional in *E. coli* and certain other gram-negative organisms. The *xyl* operator is an operator that is obtained from the xylose metabolism operon from *S. xylosus,* which is a gram-positive organism. *E. coli* does not have a repressor protein that binds the *xyl* operator. Furthermore, it is generally known in the art that promoters obtained from gram-negative organisms generally do not function in gram-positive organisms (see Jarmer et al. (2001) *Microbiology* **147**:2417-2424, page 1, column 2 – a copy of this reference is enclosed herewith for the Examiner's convenience as Exhibit G). Based on the above facts, a skilled artisan would not reasonably expect a T5 promoter fused to a *xyl* operator to function as a regulatable promoter in either a gram-negative or a gram-positive organism since it would not be expected to be regulatable in the gram-negative organism and it would not be expected to be transcriptionally active in the gram-positive organism. As such, one of ordinary skill in the art would not be motivated to combine a T5 promoter with a *xyl* operator from a gram-positive organism.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of claims 1-4, 7-11, 13-15, 18-22, 24-27, 29, 32, 33, 36-40 and 42-47 under 35 U.S.C. § 103(a).

Rejection of Claims 1-4, 7-11, 13-15, 18-22, 24-29, 32, 33, 36-40 and 42-47 Under 35 U.S.C. § 103(a)

The Examiner rejects claims 1-4, 7-11, 13-15, 18-22, 24-29, 32, 33, 36-40 and 42-47 under 35 U.S.C. § 103(a) as being obvious over Bujard et al. in view of Sizemore, et al. and in further view of U.S. Patent No. 4,959,311 (Shih et al.), U.S. Patent No. 4,656,136 (Kisumi et al.) or Pederson et al. (*Mol. Gen. Genet.* 244:374-382). In particular, the Examiner reiterates the rejection based on the combination of Bujard et al. and Sizemore et al. and then further asserts that Shih et al., Kisumi et al. and Pederson et al. allegedly "disclose, respectively, the p15a, pC194 and pCT1138 replicons and their usefulness in cloning and expression plasmids." The Examiner then asserts that a skilled artisan would have been motivated to combine the above references in order "to utilize such known replicons in order to take advantage of the known replication properties in the microorganism of interest."

Applicants maintain that claims 1-4, 7-11, 13-15, 18-22, 24-29, 32, 33, 36-40 and 42-47 are not obvious since the combination of Bujard et al. and Sizemore et al. fails to teach every element of the above-rejected claims and because the disclosures of Shih et al, Kisumi et al. and Pederson et al. do not provide the missing element. In particular, Shih et al, Kisumi et al. and Pederson et al. do not disclose "at least one nucleotide sequence modification which alters the transcriptional activity of said promoter in at least one gram-positive organism."

In addition to the foregoing, Applicants submit that claims 1-4, 7-11, 13-15, 18-22, 24-29, 32, 33, 36-40 and 42-47 are not obvious because, based on the above-cited combinations of references, a skilled artisan would neither be motivated to construct the claimed promoter/operator fusions nor would they expect such fusions to regulate transcription in an organism. In particular, the references Shih et al., Kisumi et al. and Pederson et al. do not teach or suggest that a combination of a T5 promoter with a *xyl* operator would be a construct that would have a regulatable transcriptional activity in an organism. In fact, the general knowledge in the art, as exemplified by Jarmer et al. (Exhibit G), suggests that such constructs would not possess regulatable transcriptional activity in either gram positive or gram negative organisms.

As such, the combination of the above-cited references does not render the subject matter of claims 1-4, 7-11, 13-15, 18-22, 24-29, 32, 33, 36-40 and 42-47 obvious.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of claims 1-4, 7-11, 13-15, 18-22, 24-29, 32, 33, 36-40 and 42-47 under 35 U.S.C. § 103(a).

Rejection of Claims 1-11, 13-27, 29, 32-40 and 42-47 Under 35 U.S.C. § 103(a)

The Examiner rejects claims 1-11, 13-27, 29, 32-40 and 42-47 under 35 U.S.C. § 103(a) as being obvious over Bujard et al. in view of Sizemore, et al and in further view of U.S. Patent No. 5,362,646 (the '646 patent). In particular, the Examiner reiterates the rejection based on the combination of Bujard et al. and Sizemore et al. and then further asserts that the '646 patent allegedly discloses nucleic acids which comprise a coliphage T promoter linked to *lacO* and further discloses that such fusion constructs can contain two operators. The Examiner then asserts that one of ordinary skill in the art would have been motivated to combine the above references so as implement two operator embodiments of the claimed fusion promoters in order to obtain additional expression control.

Applicants maintain that claims 1-11, 13-27, 29, 32-40 and 42-47 are not obvious since the combination of Bujard et al. and Sizemore et al. fails to teach every element of the above-rejected claims and because the disclosure of the '646 patent does not provide the missing element. In particular, the '646 patent does not disclose "at least one nucleotide sequence modification which alters the transcriptional activity of said promoter in at least one gram-positive organism."

In addition to the foregoing, Applicants submit that claims 1-11, 13-27, 29, 32-40 and 42-47 are not obvious because, based on the above-cited combinations of references, a skilled artisan would neither be motivated to construct the claimed promoter/operator fusions nor would they expect such fusions to regulate transcription in an organism. In particular, the '646 patent does not teach or suggest that a combination of a T5 promoter with a *xyl* operator would be a construct that would have a regulatable transcriptional activity in an organism. In fact, the general knowledge in the art, as exemplified by Jarmer et al. (Exhibit G), suggests that such constructs would not possess regulatable transcriptional activity in either gram positive or gram

negative organisms. As such, the combination of the above-cited references does not render the subject matter of claims 1-11, 13-27, 29, 32-40 and 42-47 obvious.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of claims 1-11, 13-27, 29, 32-40 and 42-47 under 35 U.S.C. § 103(a).

Rejection of Claims 1-4, 7-11, 13-15, 18-22, 24-27, 29-33, 36-40 and 42-47 Under 35 U.S.C. § 103(a)

The Examiner rejects claims 1-4, 7-11, 13-15, 18-22, 24-27, 29-33, 36-40 and 42-47 under 35 U.S.C. § 103(a) as being obvious over Bujard et al. in view of Sizemore, et al and in further view of Israelson et al. (*Appl. Environ. Microbiol.* **61**: 2540-2547). In particular, the Examiner reiterates the rejection based on the combination of Bujard et al. and Sizemore et al. and then further asserts that Israelson et al. allegedly disclose the *lacL-lacM* reporter gene from *Leuconostoc mesenteroides*. The Examiner then asserts that one of ordinary skill in the art would have been motivated to combine the above references so as obtain the claimed fusion promoter/reporter gene constructs "in order to obtain information regarding the level of expression of the promoter region."

Applicants maintain that claims 1-4, 7-11, 13-15, 18-22, 24-27, 29-33, 36-40 and 42-47 are not obvious since the combination of Bujard et al. and Sizemore et al. fails to teach every element of the above-rejected claims and because the disclosure of Israelson et al. does not provide the missing element. In particular, Israelson et al. do not disclose "at least one nucleotide sequence modification which alters the transcriptional activity of said promoter in at least one gram-positive organism."

In addition to the foregoing, Applicants submit that claims 1-4, 7-11, 13-15, 18-22, 24-27, 29-33, 36-40 and 42-47 are not obvious because, based on the above-cited combinations of references, a skilled artisan would neither be motivated to construct the claimed promoter/operator fusions nor would they expect such fusions to regulate transcription in an organism. In particular, the reference Israelson et al. does not teach or suggest that a combination of a T5 promoter with a *xyl* operator would be a construct that would have a regulatable transcriptional activity in an organism. In fact, the general knowledge in the art, as exemplified by Jarmer et al. (Exhibit G), suggests that such constructs would not possess regulatable transcriptional activity in either gram positive or gram negative organisms. As such, the

combination of the above-cited references does not render the subject matter of claims 1-4, 7-11, 13-15, 18-22, 24-27, 29-33, 36-40 and 42-47 obvious.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of claims 1-4, 7-11, 13-15, 18-22, 24-27, 29-33, 36-40 and 42-47 under 35 U.S.C. § 103(a).

CONCLUSION

Applicants believe that all outstanding issues in this case have been resolved and that the present claims are in condition for allowance. Nevertheless, if any undeveloped issues remain or if any issues require clarification, the Examiner is invited to contact the undersigned at the telephone number provided below in order to expedite the resolution of such issues.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: Nov. 23, 2004

By: _____

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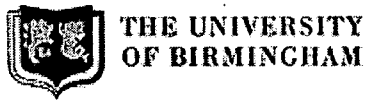

Plasmid Data Page

Plasmid: p15A

Original Host:	Escherichia coli
Host Range:	Unknown
Isolation Date:	1968
Geographical Location:	
Sample Type:	Lab isolate
Size (kb) :	2
Transfer:	U
Mobilisable:	
Reference:	Cozzarelli, N. R., Kelly, R. B., Kornberg, A. (1968). A Minute Circular DNA from Escherichia coli. Proc. Natl. Acad. Sci. 60:992-999.

Incompatibility Groups	Phenotypes	General Phenotypes
unspecified	No Phenotype (Cryptic Plasmid)	No Phenotype (or Unknown)

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EXHIBIT A

pLysS & pLysE

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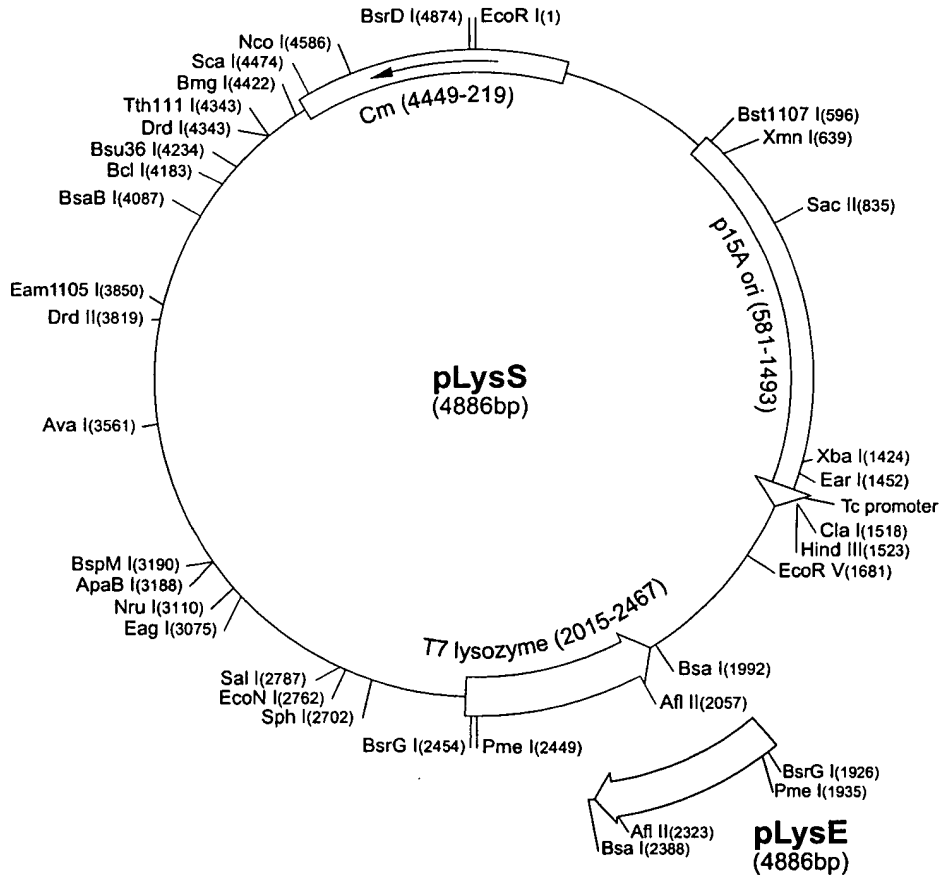
pLysS (Cat. No. 69659-3) and pLysE (Cat. No. 69658-3) are 4886bp plasmids constructed by insertion of the T7 lysozyme gene into the *Bam*H I site of pACYC184 (1, 2). These plasmids are not cloning vectors; they are used in λDE3 lysogenic hosts to suppress basal expression from the T7 promoter by producing T7 lysozyme, a natural inhibitor of T7 RNA polymerase. The two plasmids differ only by the orientation of the T7 lysozyme gene. In pLysS the T7 lysozyme coding sequence is in the antisense orientation relative to the *tet* promoter, so only a small amount of T7 lysozyme is produced. In pLysE large amounts of T7 lysozyme are produced from the *tet* promoter. The construct also contains the weak T7 ϕ3.8 promoter immediately following the lysozyme gene. The p15A origin of replication is compatible with those found in pBR322- and pUC-derived plasmids. Unique sites are shown on the circle map.

1. Studier , F.W. (1991) *J. Mol. Biol.* **219**, 37-44.
2. Chang, A.C.Y. and Cohen, S.N. (1978) *J. Bacteriol.* **134**, 1141.

pLysS & pLysE sequence landmarks

Cm gene coding seq.	4449-219
p15A origin	581-1493
T7 lysozyme coding seq. (pLysS)	2015-2467
T7 lysozyme coding seq. (pLysE)	1918-2370



pLysS Restriction Sites

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Enzyme	# Sites	Locations				
AccI	2	595	2788			
AceIII	2	2833	4872			
AciI	57					
AflII	1	2057				
AgeI	4	669	992	3823	4283	
AluI	13					
AlwI	6	1864	1877	2506	2519	3241
		4090				
Alw21I	5	494	1774	2727	3314	3605
AlwNI	3	537	1185	4165		
ApaBI	1	3188				
ApoI	3	1	4349	4361		
AvaI	1	3561				
AvaII	6	2505	2935	3023	3272	3575
		3617				
BamHI	2	1869	2511			
BanI	11					
BanII	2	2611	2625			
BbsI	4	2114	2866	3729	3960	
BbvI	16					
BccI	10	477	2100	2276	2596	2689
		3126	3215	3522	3534	4514
Bce83I	4	1158	1373	2821	2991	
BcefI	6	78	572	828	2746	3303
		4538				
BcgI	6	2143	2177	2833	2867	3927
		3961				
BclI	1	4183				
BfaI	4	584	1425	1724	3625	
BglI	2	3071	3305			
BmgI	1	4422				
BpmI	4	478	2968	3522	4771	
Bpu10I	4	229	2255	3717	4220	
BsaI	1	1992				
BsaAI	2	312	3785			
BsaBI	1	4087				
BsaHI	6	2086	2229	2550	2571	2685
		3342				
BsaJI	20					
BsaWI	11					
BscGI	11					
BseRI	3	1216	1259	1899		
BsgI	2	650	4050			
BsiI	2	959	2458			
BsiEI	6	740	1109	1783	2792	3078
		4342				
BsII	22					
BsmI	3	14	3495	4493		
BsmAI	5	330	1029	1992	2311	4663
BsmBI	3	330	2311	4663		
BsmFI	8	385	505	2518	2688	3009
		3234	3762	4001		
BsoFI	38					
Bsp24I	6	1239	1271	2383	2415	2517
		2549				
Bsp1286I	8	494	1774	2611	2625	2727
		3314	3605	4424		
BspEI	2	5	4079			
BspGI	3	1076	3195	3272		
BspMI	1	3190				
BsrI	17					
BsrBI	2	757	3866			
BsrDI	1	4874				
BsrFI	12					
BsrGI	1	2454				
Bst1107I	1	596				
BstYI	3	1869	2511	4082		
Bsu36I	1	4234				
Cac8I	33					
CjeI	22					
ClaI	1	1518				
CviJI	84					

Enzyme	# Sites	Locations				
CviRI	18					
DdeI	11					
DpnI	16					
DraI	3	85	2449	4632		
DrdI	1	4343				
DrdII	1	3819				
DsaI	7	832	2016	2664	3583	4025
		4207	4586			
EaeI	8	1013	1789	2535	2667	3075
		3580	4022	4622		
EagI	1	3075				
Eam1105I	1	3850				
EarI	1	1452				
EciI	3	780	861	3531		
Eco47III	5	582	1728	2632	2913	4144
Eco57I	2	1484	3813			
EcoNI	1	2762				
Eco0109I	5	2505	2660	3575	3617	4030
EcoRI	1	1				
EcoRII	12					
EcoRV	1	1681				
FauI	10	18	1668	2832	3034	3181
		3376	3626	3809	3926	4460
FokI	7	21	1592	1640	3136	3181
		4109	4361			
FspI	4	1756	1944	3494	3592	
GdiII	6	1013	1789	2535	2667	3075
		4022				
HaeI	7	3056	3128	3185	3582	4232
		4624	4711			
HaeII	13					
HaeIII	25					
HgaI	12					
HhaI	30					
Hin4I	6	16	406	1510	1828	3277
		3849				
HincII	2	2789	3855			
HindIII	1	1523				
HinfI	11					
HphI	16					
MaeII	10	311	323	2441	3037	3093
		3682	3706	3784	4627	4802
MaeIII	16					
MboII	13					
MmeI	5	279	1129	1168	1716	1803
MnlI	34					
MscI	2	3582	4624			
MseI	16					
MslI	4	289	3167	3598	4072	
MspI	36					
MspA1I	9	105	517	834	1006	1111
		1981	3277	3906	4547	
MwoI	39					
NarI	5	2086	2550	2571	2685	3342
NciI	11					
NcoI	1	4586				
NgoAIV	5	2537	2905	3065	3419	3999
NheI	2	583	1723			
NlaIII	24					
NlaIV	28					
NruI	1	3110				
NspI	2	1161	2702			
NspV	2	1319	4359			
Pfl1108I	2	2894	3988			
PflMI	4	343	3457	3506	4662	
PleI	4	718	1148	2776	3859	
PmeI	1	2449				
PshAI	2	2852	3946			
Psp5II	3	2505	3575	3617		
Psp1406I	2	3037	4802			
PvuII	2	105	517			
RcaI	2	2094	2625			

Enzyme	# Sites	Locations				
RleAI	2	1908	2348			
RsaI	6	126	1659	2124	2302	2456
		4474				
SacII	1	835				
SalI	1	2787				
Sau96I	13					
Sau3AI	16					
ScaI	1	4474				
ScrFI	23					
SfaNI	16					
SfcI	2	1632	2269			
SgrAI	2	669	2546			
SphI	1	2702				
SspI	2	1420	4579			
StyI	2	3505	4586			
TaqI	14					
TaqII	3	726	2806	4669		
TfiI	7	2368	2436	2988	3142	3440
		3661	4538			
ThaI	19					
TseI	16					
Tsp45I	9	409	1258	1618	1706	2027
		2138	3016	3283	4256	
Tsp509I	16					
Tth111I	1	4343				
Tth111II	3	888	1491	2159		
UbaJI	22					
VspI	2	1406	2008			
XbaI	1	1424				
XmnI	1	639				

Enzymes that do not cut pLysS or pLysE:

AatII	AflIII	Alw44I	ApaI	AscI
AvrII	BaeI	BglII	Bpu1102I	BsaXI
BsbI	BspLU11I	BssHII	BstEII	BstXI
DraIII	FseI	HgiEII	HpaI	KpnI
MluI	MunI	NdeI	NotI	NsiI
PacI	PmlI	PstI	PvuI	RsrII
SacI	SapI	SexAI	SfiI	SgfI
SmaI	SnaBI	SpeI	SrfI	Sse8387I
StuI	SunI	SwaI	XcmI	XhoI

Plasmid Data Page

PLASMID DATA PAGE

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Plasmid: pC194

Original Host:	Staphylococcus aureus
Host Range:	Narrow Gram Positive
Isolation Date:	
Geographical Location:	
Sample Type:	
Size (kb) :	
Transfer:	+
Mobilisable:	
Reference:	Iordanescu, S., Surdeano, M., Della Latta, D., Novick, R. (1978). Incompatibility and Molecular Relationships Between Small Staphylococcal Plasmids Carrying The Same Resistance Marker. Plasmid. 1:468-479

Incompatibility Groups	Phenotypes	General Phenotypes
H	Chloramiphenicol resistance	Resistance

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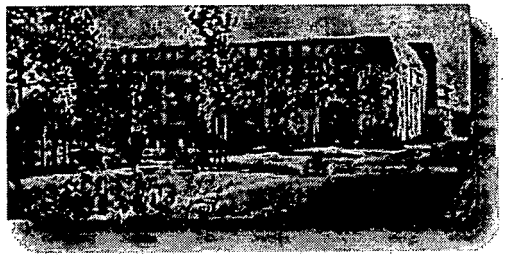

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EXHIBIT D

Plasmid pC194

DSMZ

Name	pC194
DSM No.	4393
=	SB202
History	<- S.D. Ehrlich
Mol. Weight	2.91 kb
Marker	Cm^r
Remarks	Originally isolated from *Staphylococcus aureus*; sequence known; inducible Cm resistance; use: the resistance gene, which is expressed in *E. coli*, has been used in *B. subtilis*/*E. coli* shuttle vectors and in integrative plasmids used to map cloned *B. subtilis* genes.
Distributed in	*Bacillus subtilis* subsp. *subtilis* SB202 (derivative of strain 168)
Medium	381 , 30°C
References	3169, 3170, 3171
Price	EURO 38 (non-profit making institutions), EURO 54 (other institutions): Normal price.
Restrictions	no

DSMZ List of Plasmids Microorganisms

EXHIBIT D

1: L27067. Reports Plasmid pCT1138 o...[gi:496171]

```
LOCUS       P11ORI                   181 bp    DNA     linear   BCT 22-AUG-1994
DEFINITION  Plasmid pCT1138 origin of replication (ori).
ACCESSION   L27067
VERSION     L27067.1  GI:496171
KEYWORDS    .
SOURCE      Plasmid pCT1138
  ORGANISM  Plasmid pCT1138
            other sequences; plasmids.
REFERENCE   1  (bases 1 to 181)
  AUTHORS   Pedersen,M.L., Arnved,K.R. and Johansen,E.
  TITLE     Genetic analysis of the minimal replicon of the Lactococcus lactis
            subsp. lactis biovar diacetylactis citrate plasmid
  JOURNAL   Mol. Gen. Genet. 244 (4), 374-382 (1994)
  MEDLINE   94359460
   PUBMED   8078463
COMMENT     Original source text: Plasmid pCT1138 (plasmid Plasmid pCT1138,
            kingdom Prokaryotae) DNA.
FEATURES             Location/Qualifiers
     source          1..181
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                     /mol_type="genomic DNA"
                     /db_xref="taxon:35413"
                     /plasmid="Plasmid pCT1138"
     repeat_region   5..15
     repeat_region   18..27
     repeat_region   85..106
     repeat_region   107..128
     repeat_region   129..140
ORIGIN
        1 cctattatat atttatcata tatattttaa tcttttgttc ttttgcgtga aaaaaaaggc
       61 agtgttttcg ctagttatag aaattaaaca gtcacaaaaa tcgatgtata gagtcacaaa
      121 aatcgatgta tagagtcaca aaaatcgatg tacacagcac gacttttgta tttgtgtact
      181 g
```

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EXHIBIT F

Plasmid Citrate plasmid

DSMZ

Name	Citrate plasmid
DSM No.	4366
History	<- H. Neve, Federal Dairy Research Centre, Kiel
Mol. Weight	8 kb
Marker	Citrate utilization
Remarks	The strain carries different plasmids: 72.7 kb, 25.8 kb, 15.2 kb, 8 kb (citrate plasmid), 6.8 kb, 6.1 kb; the plasmids were isolated from a starter culture; the citrate plasmid encodes citrate utilization; strain F7/2 is a host for virulent bacteriophages of lactic streptococci.
Distributed in	*Lactococcus lactis* subsp. *lactis* F7/2
Medium	449 , 30°C
References	3463, 3464
Price	EURO 38 (non-profit making institutions), EURO 54 (other institutions): Normal price.
Restrictions	no

DSMZ List of Plasmids Microorganisms

EXHIBIT F

Microbiology (2001), 147, 2417–2424 Printed in Great Britain

Sigma A recognition sites in the *Bacillus subtilis* genome

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A hidden Markov model of σ^A RNA polymerase cofactor recognition sites in *Bacillus subtilis*, containing either the common or the extended −10 motifs, has been constructed based on experimentally verified σ^A recognition sites. This work suggests that more information exists at the initiation site of transcription in both types of promoters than previously thought. When tested on the entire *B. subtilis* genome, the model predicts that approximately half of the σ^A recognition sites are of the extended type. Some of the response-regulator aspartate phosphatases were among the predictions of promoters containing extended sites. The expression of *rapA* and *rapB* was confirmed by site-directed mutagenesis to depend on the extended −10 region.

Keywords: sigma factor, HMM, response regulator aspartate phosphatase, extended −10 region

INTRODUCTION

To initiate transcription, RNA polymerase (RNAP) has to recognize and bind to the promoter region. In prokaryotic cells this ability resides in the 'specificity' (in Greek s = σ) factor of the RNAP complex. The genome of *Bacillus subtilis* encodes at least 17 different σ factors (Huang & Helmann, 1998). Growing cells utilize at least six different σ factors: the housekeeping σ^A, and σ^B, σ^C, σ^D, σ^H and σ^L, and *B. subtilis* uses yet another four during endospore formation: σ^E, σ^F, σ^G and σ^K. The remaining seven σ factors were identified after sequencing of the complete genome and are all of the extracytoplasmic function (ECF) subfamily (Huang *et al.*, 1998).

The σ factor in the RNAP complex recognizes and binds a specific conserved DNA pattern upstream of the transcription start site, thereby allowing the RNAP to associate with the DNA strand, first loosely in a 'closed promoter–polymerase complex', and then tightly, melting a local region of the promoter to form an 'open promoter–polymerase complex', resulting in initiation of transcription. When the transcription is initiated, the σ factor is released from the complex.

Every σ factor facilitates binding of the RNAP complex by recognition of a specific binding site, usually located 10 and 35 bp upstream of the transcription start site. σ^A in *B. subtilis* participates in the initiation of transcription of most of the housekeeping genes. The consensus sequence recognized by σ^A, 5′-TTGACA-17 nt-TATAAT-3′, is identical to the consensus that σ^{70} of *Escherichia coli* recognizes. σ^A-dependent promoters from *B. subtilis* are easily transcribed by the σ^{70} of *E. coli*, but poorly the other way around (Camacho & Salas, 1999), which suggests that the RNAP of *B. subtilis* has a stricter requirement for binding than the RNAP of *E. coli* (Voskuil & Chambliss, 1998; Camacho & Salas, 1999). This corresponds with the fact that earlier studies have shown that many Gram-positives including *B. subtilis* utilize an extended −10 region in a large number of their σ^A-dependent promoters. This region is located 1 bp upstream of the −10 region and is hence referred to as the −16 region. The consensus of this region is 5′-TRTG-3′, where R = G/A (Helmann, 1995; Voskuil & Chambliss, 1998; Camacho & Salas, 1999), and it is therefore larger than the corresponding 5′-TG-3′ motif found in *E. coli* (Ponnambalam *et. al.*, 1986; Keilty & Rosenberg, 1987). This extension is estimated to exist in less than 10% of the promoters in *E. coli* (Chan *et al.*, 1990) and approximately 45% in *B. subtilis*. Especially in promoters containing this extended signal a series of A- and T-rich regions upstream of the −35 region has been observed. And both σ^A-dependent promoter types have an overrepresentation of A residues downstream of the −10 region (Voskuil & Chambliss, 1998). By

Abbreviations: FP, false positive; HMM, hidden Markov model; RNAP, RNA polymerase; TP, true positive.

extracting this information it is possible to create a model for prediction of new sites.

The complete genome sequence (4·2 Mb) of *B. subtilis* was published in November 1997 (Kunst *et al.*, 1997), and at present 4228 genes are annotated (SubtiList, 1999). Approximately one-third of these genes have experimentally identified functions. The function of the second third can be predicted by homology to other known gene products. Among the last third of the genes there is most likely an unknown number of misclassified ORFs, and therefore the exact number of genes in *B. subtilis* remains unknown. It is therefore also not possible to estimate how many promoters exist in the genome of *B. subtilis*. If the annotated genes are correct and if only regions upstream of a gene and downstream of a terminator, or regions between genes arranged head-to-head, are defined as promoter regions, a conservative estimation will be that *B. subtilis* has 1800 promoter regions. The fraction of these that is dependent on σ^A is not known.

There are currently no publicly accessible tools for the prediction of σ^A-binding sites in *B. subtilis*. Nobody has attempted to estimate the number of such sites. We have used hidden Markov models (HMMs) and trained them to recognize σ^A-binding sites in *B. subtilis* from existing experimentally generated data. The goal of this work is to create a tool to predict the number of true signals within the genome. This work has the further aim of recovering possible hidden information in the surrounding sequence of the two types of σ^A-binding sites as they are known today. This will clarify the differences between the sites, and make it easier to distinguish between them.

METHODS

Hidden Markov models. The central idea of an HMM is to embed the statistics of a motif in a set of states with transitions between them. Each HMM state has a specific probability distribution over the four nucleotides and hence one may say that it 'emits' nucleotides according to specific emission probabilities. There is a state for each position in the motif and the emission probabilities essentially end up being equal to the nucleotide frequencies at these positions. Hence, an HMM may be viewed either as a generative model which 'emits' nucleotides according to specific statistics or as a scoring model which may be used to answer questions such as: 'To what extent is a given sequence compatible with/similar to the sequences used to train the HMM?'. These two HMM interpretations are equally valid and the choice between them depends on the application in question (for further introduction to HMMs we recommend Durbin *et al.*, 1998).

HMMs are generally well suited for searching for motifs like σ^A-binding sites since they facilitate an easy and intuitive incorporation of prior knowledge about signals associated with the motif in question. Another advantage, compared to techniques such as neural networks, is the ease of relating trained model parameters to sequence information; for instance, it is possible to use the trained emission probabilities to directly read off any consensus signals found and to get a good idea of the information present in these signals.

Prior knowledge may be included in the HMM architecture by addition or deletion of states, by biasing their nucleotide emission probabilities and/or biasing the probabilities of transitions between them.

When a model architecture has been set up, the optimal parameters are estimated by the Baum–Welch algorithm, which maximizes the likelihood of the training sequences given the model – i.e. it finds the HMM parameters which best capture the statistics of the training sequences.

The trained model is then used to analyse sequences not included in the training set. To get an idea of the model's ability to generalize, one may split the initial training set into 10 parts and then repeatedly train on nine parts and test on the remaining part, until all parts have been tested once. This is a common technique known as a 10-fold cross-validation. It provides a way of estimating the extent of expected false positives and false negatives for a given threshold, when using the model to decode new sequences.

'Decoding' is the term applied to the process of evaluating how well a sequence or sub-sequence fits a given HMM model. There are several ways to perform decoding, and we have used *posterior* decoding, where one calculates, for the ith nucleotide x_i in the query sequence x of length L, the total probability that the state π_i emitting it is state k, $P(\pi_i = k \mid x)$. Note that in general there are many paths through the model that could have emitted nucleotide x_i while in state k (i.e. for which $\pi_i = k$), so one must add the probabilities of all these parses to get the total probability. Formally, we have:

$$P(\pi_i = k \mid x) = \frac{P(x, \pi_i = k)}{P(x)} \tag{1}$$

The numerator may be written

$$P(x, \pi_i = k) = P(x_1 \ldots x_i, \pi_i = k)\ P(x_{i+1} \ldots x_L \mid x_1 \ldots x_i, \pi_i = k)$$
$$= P(x_1 \ldots x_i, \pi_i = k)\ P(x_{i+1} \ldots x_L \mid \pi_i = k) \tag{2}$$

since all observations after x_i depend only on π_i. The first and second term in this product may be calculated recursively by the forward and backward algorithm respectively (Durbin *et al.*, 1998). The remaining unknown on the right-hand side of equation (1), $P(x)$, may also be obtained from the forward/backward algorithms.

HMM prediction. For predicting sites in the genome we calculate, for each nucleotide, the posterior probability that it was emitted by the first state of the −10 region in a σ^A-binding motif. Once we have the posterior probabilities of the −10 start-states at all nucleotide positions, we simply regard all probabilities above a certain threshold (determined by the cross-validation procedure described above) as statistically significant. Hence, whenever the posterior probability of the desired motif exceeds the threshold, the model is said to have 'found' a motif at that nucleotide. The better the motif and its contextual signals fit the model, the higher the probability score, and the more confidence will be placed in the prediction.

Fig. 1 is a schematic view of the HMM used to predict σ^A promoters in *B. subtilis*. The model incorporates known information about conserved positions in σ^A binding (Helmann, 1995; Voskuil & Chambliss, 1998), and was trained to pick up additional unknown signals.

'BACKGROUND' is a state whose emission probabilities are obtained from a first-order HMM trained on the entire *B. subtilis* genome (direct strand). This represents a null model.

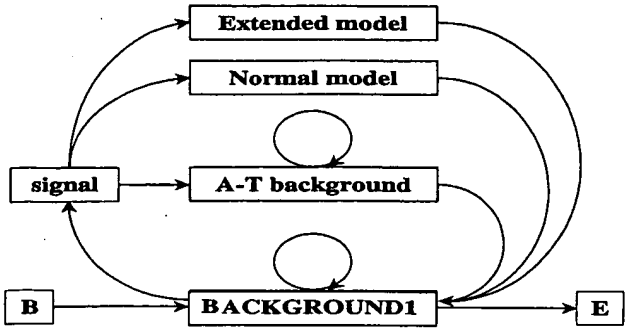

Fig. 1. A schematic drawing of the HMM used to predict σ^A-binding sites in *B. subtilis*. Each box indicates a submodel (see text). The arrows show the possible transitions between submodels. A circular arrow indicates that the model is allowed to loop (stay in the same state for more than one base in the sequence) at the given position (Durbin *et al.*, 1998).

The reason for using a low-order Markov chain for the background is to avoid modelling the genome too explicitly, since most of the genome is presumably coding whereas the promoters generally reside in non-coding regions. If the promoter finder were combined with a gene finder, one could train the background state on supposed non-coding regions and conceivably improve the signal-to-noise ratio. However, it is unclear how the promoter-finding performance would be affected in coding regions. This is certainly a possible path of further investigation.

The model shown in Fig. 1 is used exclusively for decoding (testing). For training purposes a loop model should be avoided, since in the absence of fully labelled training sequences it may end up using several motifs in its maximum-likelihood estimation even though only one is actually present; this will then distort the statistics of the motif states and hence impair decoding performance. Thus, during training a second background (identical to 'BACKGROUND') is included on the right-hand side of Fig. 1 in such a way that all three signal states must pass on to this second background and from here to the end state 'E'.

Promoter regions as well as other intergenic regions are known to be comparatively A and T rich. Hence, in order to prevent prediction of σ^A signals merely on the basis of A and T richness, a state has been added to the model ('A-T background' in Fig. 1). The 'signal' state is included purely for technical reasons in order to switch from the trained left–right model to the looping prediction model shown in Fig. 1.

Note the presence of two alternative σ^A-binding site models in Fig. 1. This is motivated by the finding of two different submodels of binding sites – one with an extended −10 region (extended) and one without (normal) (Helmann, 1995; Voskuil & Chambliss, 1998). As dictated by the data in the training set each model allows a separation of the −10 and −35 region of 16–21 bp and 4–10 bp between the −10 region and the start site of transcription (Helmann, 1995).

Fig. 2 shows a more detailed view of the extended and normal submodels. The consensus sequences of the −10 and −35 regions are clearly marked, as are the A-T-rich states. Dotted lines indicate the presence of more states than could be comfortably shown. The presence of the 9 and 5 extra explicitly modelled states in the extended model reflects the expectation that there is more information in the binding sites. The rationale behind the self-looped states is to model length distributions between e.g. the signal state (Fig. 1) and the start of the −35 region. There are two more looped states in the normal model in order to compensate for the 9 explicit states in the extended model. If the length modelling differed markedly between the extended and normal model, one would risk situations where a submodel was preferred merely on the basis of length. The emission probabilities of the looped states are identical to the background state.

Datasets. Only sequences from experimentally verified σ^A promoters were used for training and testing. We obtained these sequences from a list on John D. Helmann's worldwide web page (http://www.bio.cornell.edu/microbio/helmann/helmann.html) (Helmann, 1995) containing 236 σ^A-dependent promoters. Using a subset of these, which have supporting

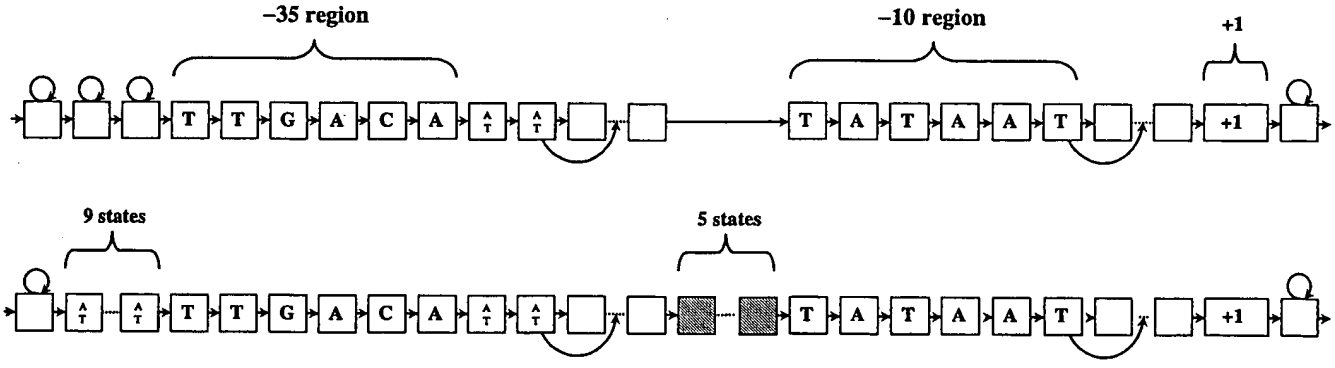

Fig. 2. A schematic drawing of the two σ^A-binding site submodels, the normal (upper) and the extended (lower). This figure uses the same symbols as Fig. 1. Background states are white without letters, the −10, −35 and +1 regions are indicated, the TG motif is hatched and other explicitly modelled states are indicated by letters. A dotted line between two boxes indicates that the number of states in this region is greater than two. An arrow pointing at the dotted line between two boxes symbolizes that the model is allowing a bypass of states. This allows the number of states between the −10 and the −35 regions to vary between 16 to 21 and likewise 4 to 10 between the −10 region and the +1 state.

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/description	Ref./source
B. subtilis		
HH263	*trpC2* (168 wild-type)	C. Anagnostopoulos*
HØJ1	*trpC2 amyE*::pB1	HH263/pB1, Neo^R
HØJ4	*trpC2 amyE*::pB4	HH263/pB4, Neo^R
HØJ5	*trpC2 amyE*::pA1	HH263/pA1, Neo^R
HØJ8	*trpC2 amyE*::pA4	HH263/pA4, Neo^R
E. coli		
MC1061	F^- *araD139* Δ(*ara–leu*)*7696 galK16* Δ(*lac*)X74 *rpsL* (Str^R) *hsdR2* ($r^- m^-$) *mcrA mcrB*	Stratagene
Plasmids		
pDG268neo	Ap^R (*E. coli*), Neo^R (*B. subtilis*); pBR322 derivative; vector used for integration of transcriptional *lacZ* fusions into the *amyE* gene	Saxild *et al.* (1996)
pB1	Ap^R (*E. coli*), Neo^R (*B. subtilis*); *Eco*RI–*Bam*HI PCR fragment containing the wild-type promoter of *rapB* (260 bp, 23 bp upstream of the ORF) cloned into pDG268	This work
pB4	As pB1 with a base substitution G to C (in the extended −10 region 42 bp upstream of the ORF)	This work
pA1	Ap^R (*E. coli*), Neo^R (*B. subtilis*); *Eco*RI–*Bam*HI PCR fragment containing the wild-type promoter of *rapA* (93 bp, 27 bp upstream of the ORF) cloned into pDG268	This work
pA4	As pA1 with a base substitution G to A (in the extended −10 region, 43 bp upstream of the ORF)	This work

* C. Anagnostopoulos, INRA, Jouy en Josas, France.

experimental data, and which are labelled at the transcription start sites (109), combined with some (11) determined in our laboratory (H. H. Saxild, unpublished results) and some (10) found in existing literature (Huang *et al.*, 1998; Huang & Helmann, 1998; Lewis *et al.*, 1998; SubtiList, 1999; Zhang & Begley, 1991), a list of 130 σ^A-dependent promoters was constructed. The 130 sequences are 100 bp long and range from approximately −85 to +15 relative to the transcription start site.

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strains HØJ1, HØJ4, HØJ5 and HØJ8 have a single-copy *rap–lacZ* transcriptional fusion inserted by a double-crossover recombination event at the *amyE* locus, with and without a base substitution in the extended −10 region. Cells were grown at 37 °C as described previously (Saxild *et al.*, 1995). Spizizen salt-buffered minimal medium supplemented with 100 μg L-tryptophan ml^{-1} was used in the enzyme assay and Luria–Bertani (LB) broth was used as rich medium. The relevant antibiotics were used at the following concentrations: neomycin, 5 μg ml^{-1}; ampicillin, 50 μg ml^{-1}.

DNA manipulations and genetic techniques. Chromosomal and plasmid DNA was isolated as described previously by Saxild *et al.* (1996). Treatment of DNA with restriction enzymes and T4 DNA ligase was performed as recommended by the supplier. Transformations of *E. coli* and *B. subtilis* were performed as described previously by Saxild *et al.* (1996). DNA sequencing was performed by the chain-termination reaction method using dideoxyribonucleotides as described by Sanger *et al.* (1977) using the Amersham Pharmacia Biotech Thermo Sequenase radio-labelled termination cycle sequencing kit. All sequencing was done with double-stranded plasmid, and was performed as described by the supplier. All PCRs were performed as described previously (Zeng & Saxild, 1999). PCR product DNAs were isolated by the use of GFX PCR DNA and gel band purification tubes from Amersham Pharmacia Biotech.

Construction of clones. The promoter regions from *rapA* and *rapB* were obtained by a PCR on chromosomal DNA from the wild-type *B. subtilis* strain 168. In addition to the wild-type promoter region, site-directed mutations were incorporated in the extended −10 region by using PCR primers with mismatches. The amplified promoter fragments with a 5′ *Eco*RI linker and a 3′ *Bam*HI linker were cloned in a transcriptional fusion with the reporter gene *lacZ* using the vector pDG268neo. The plasmid was amplified in *E. coli* MC1061, linearized with *Kpn*I and transformed into *B. subtilis* HH263. The transcriptional fusion was integrated into the *amyE* gene by a double-crossover event (Saxild *et al.*, 1996). All strains containing a transcriptional fusion were confirmed by colony PCR with relevant primers, sequencing of the cloned promoter region and verification of the $AmyE^-$ phenotype, by screening for inability to produce clearing zones on LB plates containing 1% starch. Primers complementary to regions on each side of the cloned fusion were used in PCRs to confirm that no double insertion had occurred.

Primer extension. RNA was isolated from HØJ1 and HØJ5 as described by Saxild *et al.* (1995). The single-stranded DNA primer (annealing just downstream of the *Bam*HI cloning site in pDG268) was radiolabelled at the 5′ terminus using T4 polynucleotide kinase and [γ-^{33}P]ATP. The primer extension was performed by using the displayTHERMO-RT Reverse Transcriptase kit from Display Systems Biotech. The radio-labelled cDNA probes were separated on a 6% poly-

acrylamide sequencing gel next to a sequencing of pB1/pA1 with the same primer, and visualized by autoradiography.

β-Galactosidase activity assay. Growing cells were harvested by pouring 25–30 ml culture into a 50 ml centrifuge tube 1/3 full of ice, centrifuging at 7000 *g* for 5 min, washing with 10 ml of a 0·9% NaCl solution, centrifuging at 7000 *g* for 5 min, washing with 2 ml of a 0·9% NaCl solution, centrifuging at 15000 *g* for 2 min, discarding the liquid phase, gently adding 0·5 ml 30 mM phosphate buffer (pH 7·5), 1 mM EDTA and 1 mM DTT (sonication buffer) without resolving the pellet, and stored at −20 °C. The total amount of protein was determined by the Lowry method. The β-galactosidase activity assay was performed using the method of Miller (1972).

RESULTS

Fig. 3 shows the average performance of the trained model on the test sets in the cross-validation experiment. The true positive (TP) rate is the fraction of test sequences which are predicted by the model to be σ^A sites. Ideally, TP should be 1, which would correspond to a sensitivity of 100%, but this is hardly ever feasible without paying a price in terms of a high false positive (FP) rate. The FP rate is the fraction of non-σ^A sites which are nevertheless identified by the model to be σ^A sites. Hence, ideally one wants FP equal to zero, in which case the specificity of the model is 100%. Note that in order to calculate the FP rate, one really needs a set of sequences to which one is sure that σ^A does not bind. Such a set is currently difficult if not impossible to obtain, so in the absence of a better alternative we used, for each of the 10 trained models, 1000 randomly generated sequences of length 100 with a statistic equal to 'BACKGROUND' statistics.

In most classification scenarios there is a trade-off between sensitivity and specificity, and one has to find a balance (by choosing a threshold) which is sensible for the application in question. From Fig. 3 it is clear that one can achieve a TP rate of about 0·7 with a very low FP rate. Thus the probability threshold corresponding to this sensitivity was our chosen threshold. Note that the false negative rate at this threshold is $1-TP = 0{\cdot}3$, meaning that 30% of all true sites are not reported.

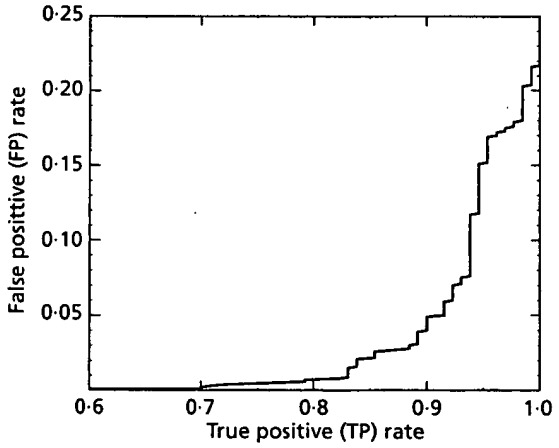

Fig. 3. The rate of false positives versus the rate of true positives.

The predicted signals always contain the whole σ^A-binding motif, and the expected transcription start site, but are reported based on the score from the −10, rather than the −35, sequence. As observed in *E. coli*, the −10 region of *B. subtilis* tends to occur unaccompanied by a −35 signal (or accompanied by an extremely poor one) in σ^A-binding promoters (Camacho & Salas, 1999), though usually dependent on an activator (Lewis *et al.*, 2000). The converse is relatively rare, and presumably such single −35 sites are not sufficient to bind σ^A and should therefore not be counted as hits.

In order to estimate the number of FPs made on the entire genome we generated 100000 random sequences of length 100 with 'BACKGROUND' statistics and counted the number of sequences scoring higher than the chosen cutoff. We did this three times and got 185, 199 and 225 sequences respectively. We then simply assume that the mean of these numbers, 203, is the expected number of FPs made on 100000 candidates. In addition to the first-order Markov statistics used in 'BACKGROUND', we also tried generating the random sequences from *k*th-order Markov chains for $k = 0$, 2 and 3. The number of sequences found on average in these cases was 791, 265 and 270 respectively – i.e. they all performed worse than the chosen $k = 1$.

Note that it is conceivable that some of the high-scoring random sequences would in fact bind σ^A in an experimental setup and hence are not really FPs. Nevertheless, this is our best estimate. In a genome of length 4·2 Mb the model is therefore expected to find roughly $(4{\cdot}2 \times 10^6/100)(203/100000) = 85$ FPs on both the positive and the negative strand, making a total of 170.

Using the HMM we predict that the entire genome of *B. subtilis* contains 2538 σ^A-binding sites. When examining the list containing the reported results (1927 high-confidence predictions) we were able to locate 1127 of these within the 400 bp upstream regions of the 4228 predicted genes in *B. subtilis* (SubtiList, 1999). Both these lists are available from the authors upon request.

The model further predicts that approximately 50% of the predicted sites are of the 'extended' type, which is a little more than previous findings on smaller samples (45%) (Helmann, 1995).

The sequence logos in Fig. 4 show the profiles of the HMM predictions. From this it is clear that more information exists in both types of σ^A-binding sites than previously thought. It is especially clear that our model has found that the transcription start site in *B. subtilis* promoters dependent on σ^A is highly conserved. The consensus sequence of this signal is 5′-YRTA-3′ (+1 in bold) in the normal type, and 5′-YRNA-3′, where Y = C/T, R = A/G and N = nt, in the extended type. The most frequent observed +1 signal in σ^A-binding

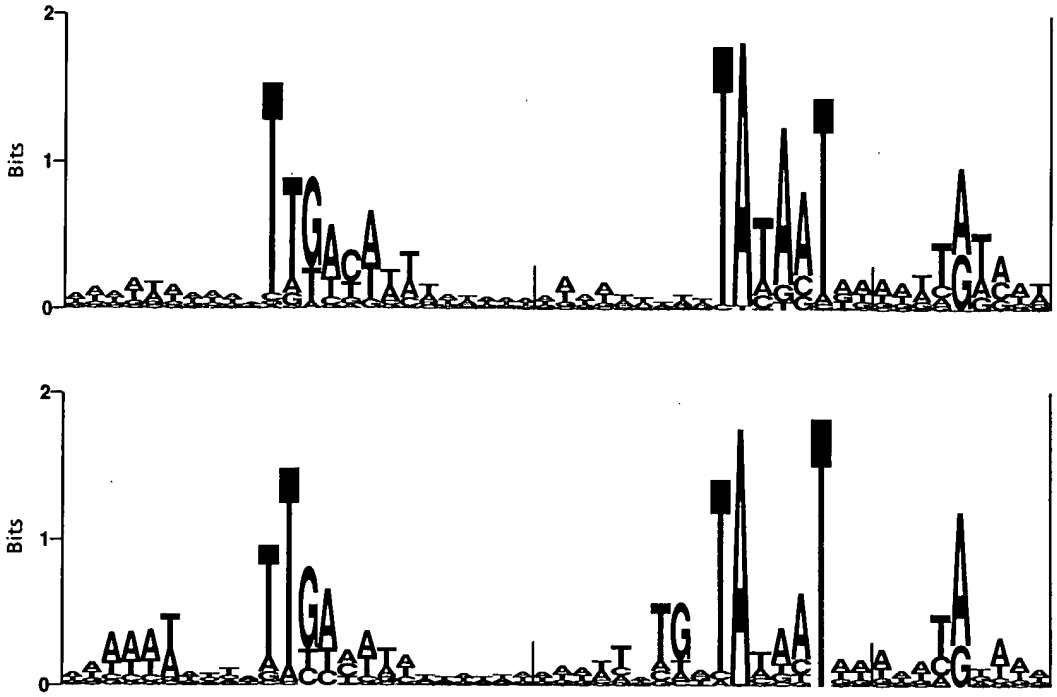

Fig. 4. Logos of the predicted σ^A-binding sites in *B. subtilis*. The logos shown are merged from six individual logos (the merging positions are shown in the figure by horizontal bars), each containing either the −10 or the −35 signal from either the normal (top logo) and the extended (bottom logo) type of σ^A recognition sites. Each of the −10 and the −35 signals represents approximately 500 signals predicted by the HMM. Each of the +1 logos is generated from 350 predicted signals. The logos are constructed by aligning the six types of signals on the first base of the reported signal. For the +1 signal, this base is represented by the highest peak in that area, with an A on the top in both types of binding site. The Shannon information content is shown on the *y* axis; Shannon's unit of non-randomness is the bit (short for 'binary digit') (Shannon, 1948).

promoters is, according to these results, 5′-TATA-3′. The signal in the +1 position is strongest in the extended type, and here an A is much more frequent than a G. This may be to compensate for the fact that the +2 position in this type of binding site is less conserved.

The model has, as expected, found that the extended type of σ^A-binding sites has an A- and T-rich area approximately 4 bp upstream of the −35 region, and that the 3′ end of the consensus of the −35 region in this type of promoters is poorly conserved. The middle section of the −10 region is likewise less conserved in the promoters of the extended type when compared to the normal type. The −16 motif is found to be 5′-TNTG-3′, which almost corresponds with the findings in other Gram-positives and previous findings in *B. subtilis* (Helmann, 1995; Voskuil & Chambliss, 1998; Camacho & Salas, 1999). Both types of promoters seem to have a slightly conserved tail with a length of 2 nt of Ts and As following the −35 region, and likewise it is found that the level of As is above average downstream of the −10 region.

The model identified σ^A-binding sites in the expected promoter regions of eight of the response-regulator aspartate phosphatase encoding genes (the *rap* genes). Our model predicts that *rapB*, *-D*, *-E*, *-I* and *-K* are transcribed from a σ^A-dependent promoter using the extended σ^A-binding site, and that *rapF*, *-G* and *-H* have the normal σ^A-binding site.

rapB	ATACATTA**TG**ATA**AAAAT**ATAACCAA
rapA	TGTAAATA**TG**ATA**AAAAT**ATGACATA
rapC	TATAAACA**TG**ATA**AAAAT**ATGACATA
rapF	AATGTTGA**TG**ATA**AAAAT**ATGACATA
rapJ	AACAGCTA**TG**ATA**AAAAT**ATAACATA
rapD	AAAAGTTA**TG**ATA**TG**ATAATTATAG
rapH	TTTGGGAT**TG**ATA**G**A**AT**ATGACATA
rapI	GGTTATTC**TG**ACA**T**A**AT**ACAATTAA
rapK	AATGACTA**TG**TTA**TG**A**T**TGTTTTCG
rapE	CGAAAACT**TG**TTA**AT**A**T**TTACAGTA
rapG	GAAAGAGG**TG**TTA**C**TA**T**CAGAATAA

Fig. 5. A multiple alignment of the expected −10 region of the *rap* genes. Fully conserved residues are shown in bold. The TG motif is observed in all the *rap* genes. The positions of transcription start are shown by underlining the first base of the transcript. The transcription starts indicated for *rapA* and *rapD* are experimentally verified (Mueller *et al.*, 1992; Huang & Helmann, 1998), the rest are solely predicted by the HMM, having a score higher than the cutoff used.

In Fig. 5 the expected −10 regions of the *rap* genes are aligned. There appears to be a highly conserved con-

Table 2. *β*-Galactosidase activity

Strain	Relevant genotype	*β*-Galactosidase activity (±SD)*
HØJ1	Wild-type	45 (±18)
HØJ4	G_{42}→C	4·1 (±2·3)
HØJ5	Wild-type	710 (±290)
HØJ8	G_{43}→A	170 (±71)

* The *β*-galactosidase activities are reported as the mean (±standard deviation) of eight independent measurements.

sensus containing a TG motif immediately upstream of a −10 motif in all the *rap* genes, which at least implies that this group of genes are being transcribed from a promoter containing an extended −10 region.

The aligned putative extended −10 region for *rapF* in Fig. 5 is not the one predicted by the model. The model predicts a −10 region 10 bp further downstream. The sequence shown in Fig. 5, however, aligns with the experimentally verified −10 region in the promoter region of *rapA* and *-D*. We suggest that *rapF* might utilize both putative σ^A-binding sites.

Experimental verification of predicted extended sites

We tested these predictions by site-directed mutagenesis of the extended region within the predicted σ^A-binding site. The site-directed mutagenesis (see Table 2) indeed showed a decrease in transcription for both *rapA* and *rapB* throughout a sporulation experiment (*rapA*, *-B* and *-E* are known to play a role in the phosphorelay signal-transduction system of sporulation: Mueller *et al.*, 1992; Jiang *et al.*, 2000), confirming that this region is necessary for transcription. When the G in the TG motif in the promoter region of *rapB* is substituted with a C, the amount of transcript drops on average 10-fold in the sporulation experiment. Likewise, when the corresponding G upstream of *rapA* is substituted with an A, the level of transcription drops approximately fourfold.

Primer extension of *rapA* confirmed two previously mapped transcription start sites (1 bp apart) (see Fig. 5) (Mueller *et al.*, 1992). For *rapB*, we were unable to detect any clear signal in repeated experiments, presumably due to the lower expression level of this gene, to instability of the messenger, or to both.

DISCUSSION

By using the HMM-based prediction tool we have constructed, we are able to predict that the genome of *B. subtilis* contains roughly 2538 σ^A-binding sites. We have generated a list containing 1127 binding sites, which are located within the 400 bp sequences upstream of predicted genes. By examining Fig. 3 it is clear that the constructed model can predict almost 70% of the true sites, virtually without predicting sites that do not actually bind the σ^A factor. It is also clear that the model can be used to predict an even larger percentile of the true sites with a low level of false positive (FPs). The model would predict only 1% FPs when predicting 83% of all true binding sites, or 7% when predicting 94%. In cases where a rate of FPs of almost 22% is acceptable, all true binding sites would theoretically be predicted.

When using the chosen cutoff, we are unable to locate approximately 30% of the true σ^A-binding sites. These false negatives are binding sites that in a variety of ways differ from the average σ^A-binding sites. One example of true σ^A-binding sites that this prediction tool has difficulties in finding are the Spo0A-activated promoters. These promoters are known to have one or several 0A boxes at or near the −35 region, where Spo0A∼P binds and activates transcription. This binding abolishes the negative effect of not only the poorly conserved −35 regions, but also the exceptionally large separation between the −10 and the −35 region (more than 21 bp), which promoters of this type are known to have (Lewis *et al.*, 2000). These sites do not fit the model due to the fact that the model only allows a spacing of 16–21 bp. Despite this drawback, we chose to accept this restriction because it gave rise to the model with the best overall performance.

The large spacing and poorly conserved −35 regions, which are often observed in activator-dependent promoters, could explain why the model does not find any true σ^A-binding sites in either *rapA*, *rapC*, *rapE*, or the putative second site in *rapF*, though there apparently exists a strong signal for an extended −10 region in this group of genes. *rapA*, *rapC* and *rapE* are known to be activated by the binding of ComA∼P to a ComA box upstream of the −35 region (Mueller *et al.*, 1992; Lazazzera *et al.*, 1999; Jiang *et al.*, 2000). When the expected promoter regions of the *rap* genes are aligned, it appears that *rapF* has a ComA box consensus site at the same position as *rapA*, *rapC* and *rapE*, which strongly suggests that expression of *rapF* is also dependent on ComA∼P (alignment not shown).

In Fig. 4 it is observed that the HMM has found a highly conserved signal at the +1 position. It appears that the site of initiation of transcription in σ^A-dependent promoters is separated from the −10 Pribnow box by on average 7 bp and has the consensus 5′-pyrimidine-purine-T-A/C-3′ (most frequent: 5′-TATA-3′), and starting transcription at the purine. This corresponds with findings in *E. coli*, where the initiation site in σ^{70}-dependent promoters is -purine-pyrimidine- (Rosenberg & Court, 1979; Pedersen & Engelbrecht, 1995).

From this work, it is suggested that the σ^A-binding sites classified as extended are significantly different from normal σ^A-binding sites in two areas of the promoter sequence. These differences are the −16-motif and the four bases approximately 40 nt upstream of the initiation site, which seem to be rich in A and T. The extended type has likewise been found to be less conserved at position 5 of the −35 region (the C in TTGACA) and at the +2 position in the +1 motif.

In conclusion, we have constructed an HMM that has identified σ^A-binding sites in *B. subtilis* with known sensitivity and specificity. We have estimated the total number of σ^A-binding sites to be around 2538, and found the ratio between extended and normal −10 regions of σ^A-binding sites to be around 1:1. To support these findings we have experimentally verified that two of the predicted promoters indeed depend on an extended type of σ^A-binding site.

The trained HMM is available from the authors upon request. The list of predictions from the trained HMM is available as supplementary data with the online version of this paper at http://mic.sgmjournals.org.

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